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The effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and *meso*-2,3-dimercaptosuccinic acid (DMSA) on the nephrotoxicity in the mouse during repeated cisplatin (CDDP) treatments

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ABSTRACT

Previously, we reported that specific lower dose of sodium 2,3-dimercapto-1-propanesulfonic acid (DMPS) which is an antidote to heavy metal intoxication, inversely enhanced cisplatin (CDDP)-induced antitumor activity to S-180 cell-bearing mouse. This activity was only weak with *meso*-2,3-dimercaptosuccinic acid (DMSA), however. This study investigated the effects of lower doses of DMPS or DMSA on the nephrotoxicity and kinetics of CDDP. Kidney and blood isolated from female mice which received CDDP with or without DMPS or DMSA once daily for 4 days were provided for measuring levels of blood urea nitrogen (BUN) and transporter proteins (OCT2: organic cation transporter; MATE1: multidrug and toxin extrusion) mRNA, and CDDP-originated platinum, and TUNEL staining of renal tubular cells. DMPS or DMSA reduced effectively CDDP-induced BUN, and caused a moderate reduction of platinum in kidney. Additionally, both dimercapto-compounds restored the CDDP-reduced mRNA levels of transporter proteins (OCT2 and MATE1), and apparently suppressed the CDDP-induced apoptosis. These results suggest that DMPS, as well as DMSA, at approximate 17-fold dose (μmol/kg) of CDDP, has an enough potential to reverse the CDDP nephrotoxicity, and concomitant use of DMPS considering both dose and timing for administration is potentially useful for preventing nephrotoxicity and enhancing antitumor activity during CDDP chemotherapy.

1. Introduction

Cisplatin (cis-diammine-dichloroplatinum; CDDP), an inorganic platinum complex has been evaluated as a most available chemotherapeutic drug for various types of malignant tumors.[1–4] However, CDDP-induced nephrotoxicity is a major limitation to CDDP-based chemotherapy.[5] The causes of the renal toxicity appeared to be due to the accumulation of CDDP in the kidneys followed by apoptosis of renal tubular cells.[6] Apoptosis induced by CDDP seems to be based on mitochondria-dependent and -independent pathways,[7] partly through the activation of caspase-3.[8] Further, CDDP-generated oxidant stress also appears to contribute to the apoptosis of renal tubular cells.[9] On the other hand, several reports offered an aspect that CDDP impaired the function of transporters on the renal tubular cell membranes to be resulted in renal cumulative uptake of CDDP.[10,11] Major transporters involved in the uptake and excretion of CDDP in the renal tubular cells are organic cation transporter (OCT) family and multi drug and toxin extrusion (MATE) family. In these transporters, OCT2 and MATE1 play significant roles in excretion of BUN or metabolites of drugs.[12–14] The OCT family is driven electrogenetically by inside-negative membrane potentials, consequently taking diverse organic cations into renal tubular cells.[15] In contrast, the MATE family, which mediates the exchange of organic cations with hydrogen ions in renal brush-

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border membranes, is suggested to be responsible for the final step of urinary excretion of cationic drugs including CDDP.[16,17]

Dithiol metal chelators such as 2,3-dimercapto-1-propanesulfonic acid (DMPS) and *meso*-2,3-dimercaptosuccinic acid (DMSA) are generally considered suitable for treating cases of heavy metal poisoning, including those caused by lead,[18] mercury,[19,20] cadmium,[21,22] and copper.[23] In the previous report, we showed that DMPS, but not DMSA, synergistically enhanced the antitumor activity of CDDP with 1.4-fold efficacy, only when two drugs were administered at an adequate dose ratio and time interval.[24] However, when DMPS enhanced the antitumor effects of CDDP, it is not clear how DMPS influenced to the nephrotoxicity. The present study, by means of examining the changes of BUN, kinetics of platinum, expression of cation transporter genes and apoptosis of renal tubular cells, investigated the ability of DMPS to protect renal tissue against CDDP-induced toxicity at lower doses which enhanced antitumor effect of CDDP, while comparing to the potency of DMSA.

2. Materials and methods

2.1. Animals

Female SPF ddy-mice weighing 25–30 g were purchased from Japan SLC (Hamamatsu, Japan). The animals were group-housed in laboratory cages with wood chips in an air-conditioned room (temperature 23 ± 2 °C, humidity 55 ± 5%, and lighting 6:00 am–6:00 pm) and maintained on commercial laboratory chow and water *ad libitum* for one week before the experiments. All things used for the animals were sterilized by autoclaving or radiation. All animals received humane care in accordance with the Guidelines for the Treatment of Experimental Animals approved by Tokyo Dental College and the Japanese Pharmacological Society. (Approval Number: 250702).

2.2. Chemicals

CDDP, DMPS and DMSA were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). PCR primers and the SUPERSCRIPT First-Strand Synthesis System for RT-PCR containing reverse transcriptase (RTase) (Superscript™ III, RNase H$^-$), dithiothreitol (DTT), oligo(dT)12–18 primer, deoxynucleosidetriphosphate (dNTP), RNase H, DNase I, and RNase inhibitor were purchased from Invitrogen (San Diego, CA, U.S.A.). NucleoSpin RNAII was obtained from MACHEREYNAGEL GmbH & Co. (Duren, Germany). The DyNAmo SYBER green qPCR Kit was obtained from Finnzymes (Espoo, Finland), and the DNA 1000 Lab Chips Kit (Agilent Technologies, Palo Alto, CA, U.S.A.) was obtained from Takara Bio Co. (Tokyo, Japan). All other reagents used were of the highest grade commercially available.

2.3. Preparation and administration of CDDP, DMPS and DMSA

CDDP was dissolved in 0.9% sodium chloride solution acidified at pH 3.0 with hydrochloride in water bath at 60 °C. DMPS or DMSA was dissolved in physiological saline or 0.9% sodium chloride solution alkalinized with 0.5% $NaHCO_3$, respectively. All agents which were prepared immediately before use were administered to each animal with a volume of 0.1 ml/10 g body weight. Untreated animals which received no drug stress were given a vehicle used for solubilizing CDDP and DMPS or DMSA.

The details of drug administration and sampling for the BUN measurement, platinum kinetics, transporter gene analysis and TUNEL staining analysis was described in Fig. 1.

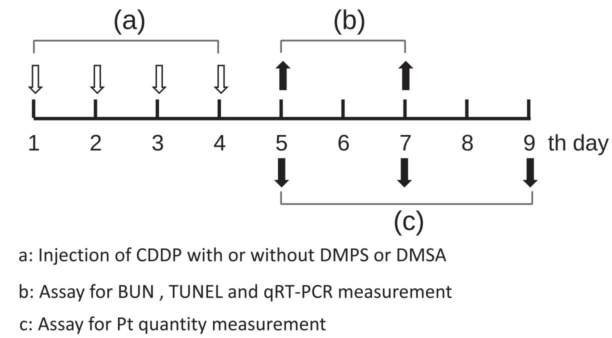

Fig. 1. Time schedule for drug administration and collecting specimen. (a) showed that drugs were repetitively administered once daily for 4 days. The administration of CDDP was performed 1 h after pretreatment with DMPS or DMSA. CDDP was administered at 5.7 μmol/kg, s.c. for each time point in all experiments. DMPS and DMSA were administered at 50, 100 and 250 μmol/kg, s.c. for the BUN measurement, whereas for other experiment, the dose was limited to 100 μmol/kg, s.c. (b) showed the sampling time for the assay of BUN, qRT-PCR and TUNEL staining and (c), for platinum isolation in kidney and plasma.

2.4. Measurement of BUN

On 5th and 7th day after the initial administration of drugs, blood sample was collected 100–150 μl through a glass capillary tube inserted into orbital venous plexus under pentobarbital anesthesia. In order to take serum more than 50 μl, these samples were centrifuged at 4 °C, for 5 min, at 12,000 g. The BUN concentration was determined by the diacetylmonoxime method using assay kits purchase from the Wako Pure Chemical Industries.

2.5. Measurement of platinum

On 5th, 7th and 9th day after the initial administration of drugs, blood sample was collected as well as in the BUN measurement, and then kidney was isolated immediately after sacrificing by over dose of anesthesia. In order to take plasma, blood samples were immediately heparinized in the ice-cold tubes and centrifuged for 10 min, 12,000 g at 4 °C. The excised kidneys and plasma were sonicated 10 min in 70% HNO_3 with 50 μg/ml Iridium (Ir) as internal standard. Each sample was diluted 500-fold by ultrapure water (Milli Q Millipore Japan). The amount of platinum was determined using the ICP-MS (inductively coupled plasma-mass spectrometry; Agilent 7500 CS, U.S.A.).

2.6. Quantitative RT-PCR

Specimen for DNA was obtained from kidney removed at 1 h, 2 h, 6 h, 12 h after the final administration with CDDP ± DMPS or DMSA. After treatment of total RNA with DNase I, first-strand cDNA was synthesized using Oligo(dT)12–18 primers and Superscript™ III RNase H–reverse transcriptase. Gene expression of OCT2 and MATE1 was determined by using the β-actin gene (GenBank accession number NM_007393.3) as an internal control and primers specific for OCT2 mRNA (accession number NM_013667.2) (upper primer, AGT GGC CTA TGC CCT TCC; lower primer, TGG AGA CTC CGG TAT GCA C; product size, 100 base pairs), MATE1 mRNA (NM_026183.5) (upper primer, GCT CTT TGC TGTGAC CTT CTG; lower primer, GAT CAC TTG AGC CAC CAA GG; product size, 106 base pairs). The cDNA was amplified by real-time quantitative PCR using the DyNAmo SYBER green qPCR Kit (Finnzymes) on the DNA Engine Opticon 2 System (Bio-Rad Laboratories; Hercules, CA, U.S.A.), running 48 cycles of the following protocol: 15 min pre-denaturation at 95 °C, 10-s denaturation at 94 °C, 20-s annealing at 59.0 °C for β-actin, OCT2, MATE1, followed by a 30-s extension at

72 °C. The PCR products were separated with the Agilent 2100 Bioanalyzer (Agilent Technologies, U.S.A.), which utilizes chip-based nucleic acid separation technology. Furthermore, identification of the amplified PCR products of the OCT2, MATE1 and β-actin cDNAs was performed by dye terminator cycle sequencing.

2.7. Evaluation of apoptosis by TUNEL staining

Kidneys were fixed in 10% formalin in PBS, embedded in paraffin, and sectioned in thickness of 4.0 μm. Each section was stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining to assess the apoptosis that is induced with tissue damage. TUNEL staining was performed using an In situ Apoptosis Detection Kit (Takara Bio, Inc., Shiga, Japan). A Zeiss Axiophot 2 microscope (Carl Zeiss Imaging Solutions, GmbH, Munich, Germany) was used for immunofluorescence microscopy (TUNEL staining). Images were captured using a chilled three-chip color charge-coupled-device camera and digitized using image analysis software (AxioVision Rel. 4.6.3.0; Carl Zeiss Imaging Solutions).

2.8. Statistical analysis

The results are given as the mean with standard deviation (S.D.) of data. When a significant difference among more than three groups was obtained in the Kruskal–Wallis *H* test, Steel–Dwass post hoc test was applied to determine the significant pairwise differences. A *P*-value <0.05 was considered as reaching statistical significance.

3. Results

3.1. The effects of DMPS and DMSA on protection against CDDP-induced renal dysfunction

CDDP increased the serum BUN levels by 87% and 61% comparing with the untreated group on both 5th and 7th day, respectively (Fig. 2A–D). DMPS at doses of 50–250 μmol/kg, i.p. prevented these changes in a dose-dependent manner with a maximum 41% decrease comparing with the CDDP alone on 7th day (Fig. 2A and B). The recovering effect in the DMPS-pretreated group indicated almost the same level of BUN as in the control group. On the other hand, DMSA completely inhibited CDDP-induced change with all doses at 50–250 μmol/kg, i.p. on both 5th and 7th day (Fig. 2C and D).

3.2. The effects of DMPS and DMSA on protection against CDDP-induced apoptosis in renal tubular cells

As shown in Fig. 3, immunohistochemical observations after staining of renal section with TUNEL made apparent apoptotic cells in the renal tubules of animals treated with CDDP (5.7 μmol/kg/day for 4 days) comparing to the untreated control (Fig. 3A and B). It was noteworthy that in the concomitant administration of DMPS or

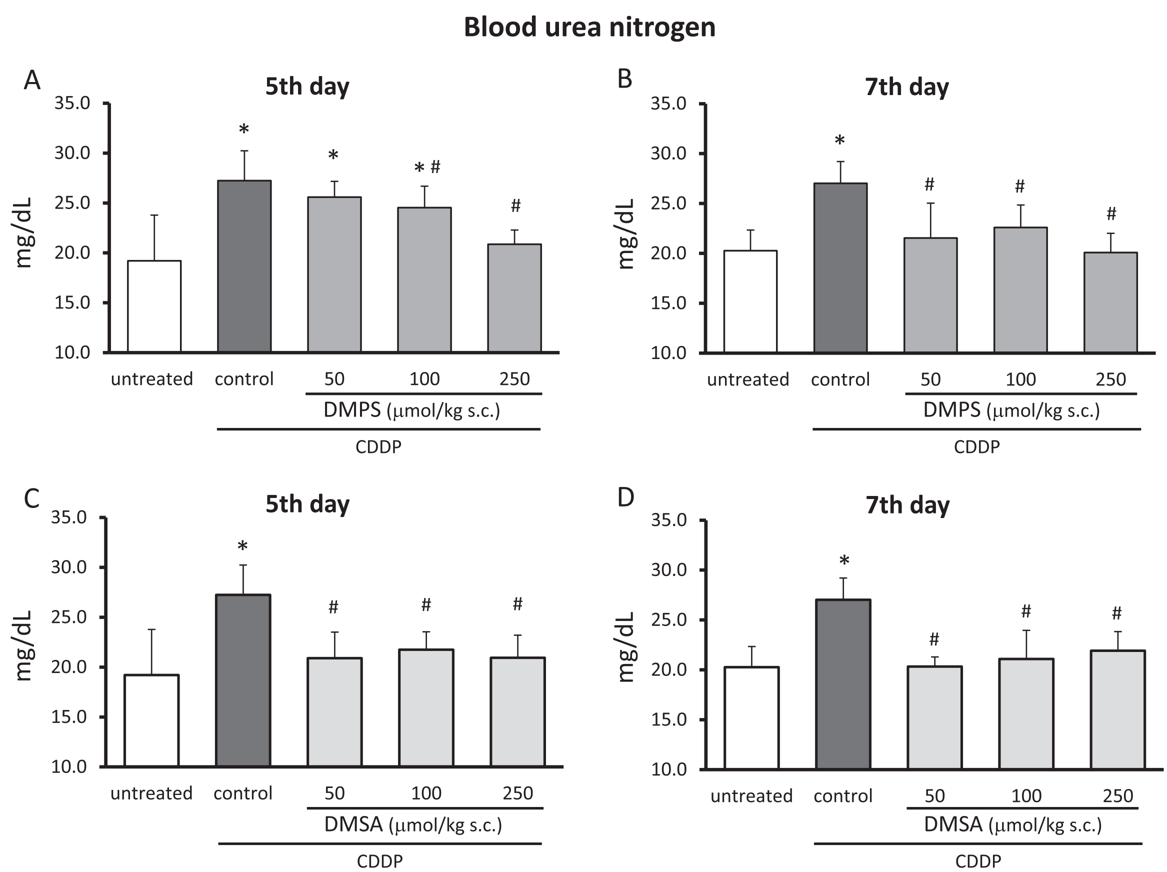

Fig. 2. Effect of DMPS (A, B) and DMSA (C, D) pretreated against the increase of BUN by CDDP. CDDP at 5.7 μmol/kg with or without both dimercapto-compounds were administered once daily for 4 days. On day 5 and 7, BUN were measured. Data were shown by mean ± S.D. of 6 mice per group. Statistically significant differences (*p < 0.05 vs the untreated group, #p < 0.05 vs the CDDP control group) are indicated.

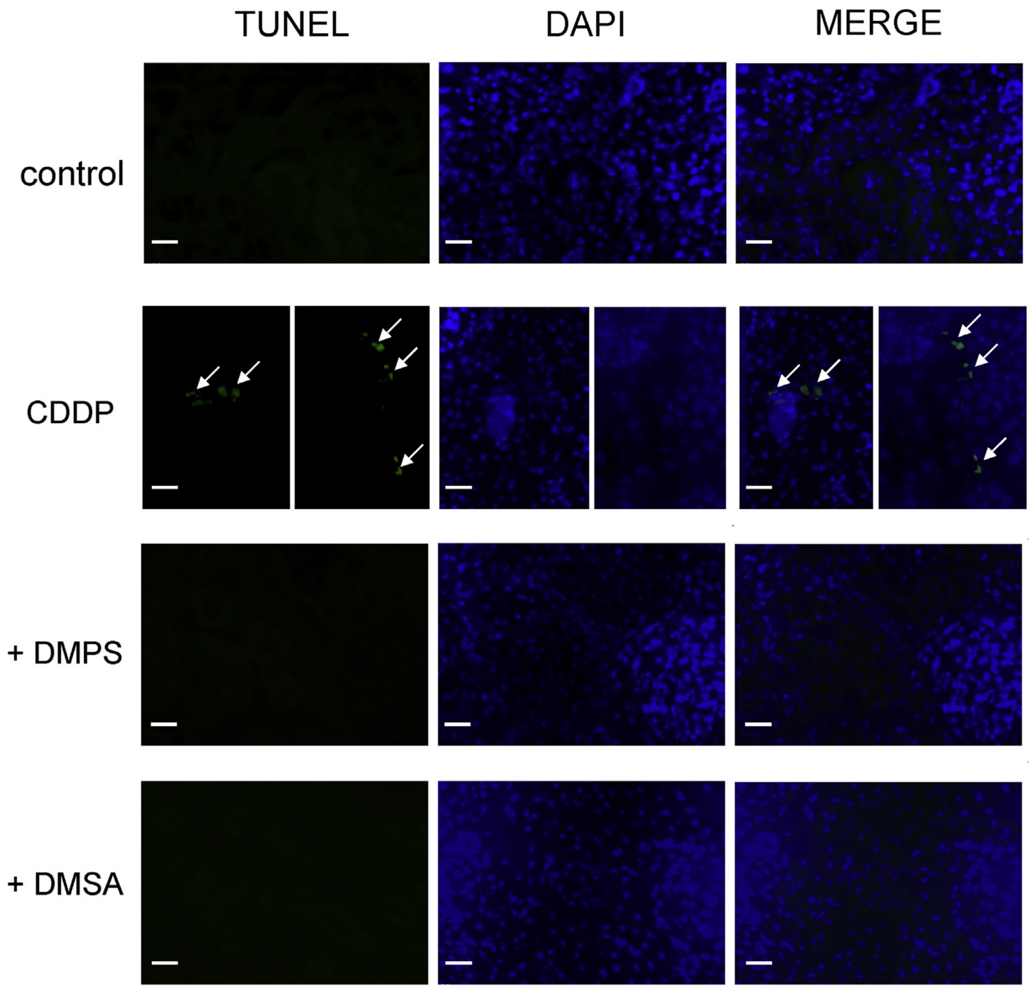

Fig. 3. Evaluation of apoptosis by TUNEL staining. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling; a cell apoptosis marker, green signal) and DAPI (4,6-diamidino-2-phenylindole; a marker of cell nuclei, blue signal) in paraffin-embedded kidney sections from each group on 7th day. TUNEL-positive nuclei were observed the CDDP alone. Scale bar: 20 μm. The number of animals used for morphological study was 3 mice for each group.

DMSA (100 μmol/kg/day for 4 days), TUNEL-stained cells were not found at all. Thus, both dimercapto-compounds revealed protective effects against the CDDP-induced apoptosis in renal tissues.

3.3. *The mRNA expression of transporters: OCT2 and MATE1*

As shown in Fig. 4, CDDP generally did not cause major changes in both transporter mRNA levels. Precisely, CDDP gave inhibitory effects with significant decrease (by 24%) in the MATE1 mRNA levels only on 5th day. Then those phenomena continued with weak suppressive tendency (around 20% lower) also on 7th day (Fig. 4B). On the other hand, the OCT2 mRNA levels were hardly affected by CDDP on both days (Fig. 4A). When DMPS and DMSA were administered concomitantly with CDDP, both mRNA levels on both days were not changed at all comparing to the untreated control. Both dimercapto-compounds completely reversed the transporter production suppressed by CDDP.

3.4. *Kinetics of platinum as CDDP in kidney and blood plasma*

CDDP 5.7 μmol/kg, s.c. singular or combination with either DMPS or DMSA (100 μmol/kg, s.c. for each) was administered once daily for 4 days. To detect accumulation of CDDP in the kidney and blood plasma, platinum was quantitatively measured by ICP-MS on 5th, 7th and 9th day. As shown in Fig. 5A, the platinum concentrations in the kidney were kept at higher levels without decrease in the CDDP alone group through the experiment. In contrast, the pretreatment with either DMPS or DMSA reduced platinum levels in a time-dependent manner with the significant decrease of 25% or 27%, respectively, on 9th day compared with on 5th day. In plasma (Fig. 5B), platinum concentrations in all of CDDP alone and DMPS- and DMSA-pretreated groups rapidly decreased in a time-dependent manner, whereas there were no significant differences between those groups in each day. Thus, it is demonstrated that DMPS and DMSA contributed to the gradual excretion of platinum from the kidney without a dramatic decrease like in plasma.

4. Discussion

This study investigated that the pretreatment with DMPS at a lower dose, which complied with the optimal dose ratio of DMPS to CDDP, produced not only the reinforcement of the anticancer activity[24] but also the protective effects, as well as DMSA, to the renal function. In the previous report, we demonstrated that when CDDP and DMPS were concomitantly administered at the adequate dose ratio and time interval, the antitumor activity of CDDP was strengthened in a maximum.[24] The maximal potentiating effects of DMPS on the antitumor activity of CDDP occurred at a dose ratio of

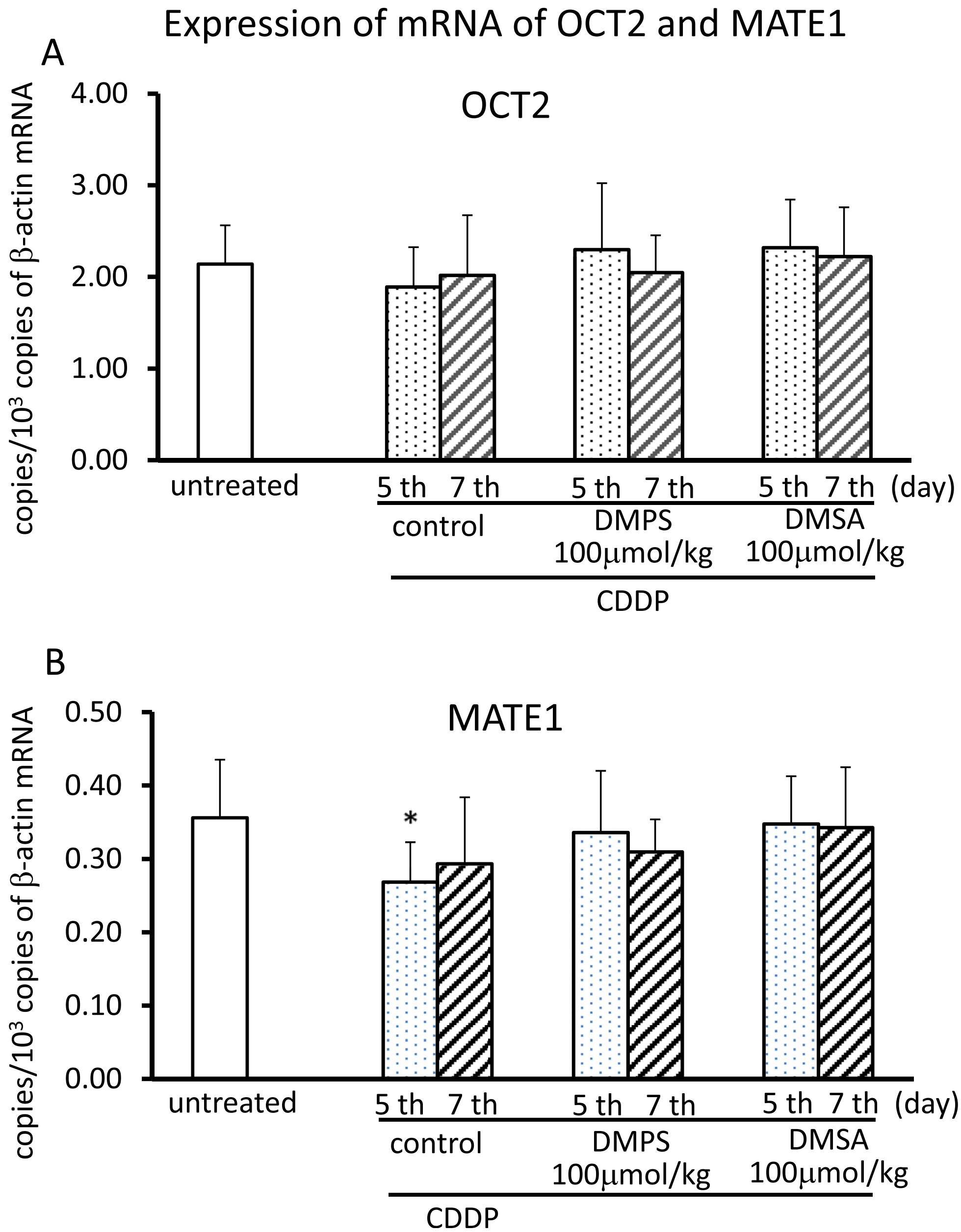

Fig. 4. The mRNA expression of OCT2 and MATE1. The mRNA expression of OCT2 (A) and MATE1 (B) on 5th and 7th day after single administration of CDDP 5.7 μmol/kg with or without DMPS, DMSA 100 μmol/kg. Data are shown by mean ± S.D. of 6 mice per group. Statistically significant differences (*$p < 0.05$ vs the untreated group) are indicated.

100 μmol/kg, s.c. to 5.7 μmol/kg, s.c., and the favorable time interval for administration of DMPS was 1 h before CDDP. On the other hand, DMSA did not show an augmentative effect on antitumor activity of CDDP.[24]

In the several researches for the purpose of reducing nephrotoxicity of CDDP, some derivatives of dimercapto-compounds have been applied for recovering of the renal function injured by CDDP.[20,25–28] DMSA and DMPS, more beneficial compounds, are featured by less toxic, easily soluble to water, more stable at the pH of the body than prototype such as dimercaprol (BAL: British Anti-Lewisite).[29] Some researches provided the effectiveness of those agents on removing platinum or palliating the severity of renal function.[25–28] As their researches were focused to extrude platinum from the kidney following the CDDP administration, the doses of dimercapto-compounds were set in much higher levels (e.g. 1000–2000 μmol/kg, i.v.)[27,28] compared with our setting (100 μmol/kg, s.c.). The group of Graziano et al succeeded in reducing renal platinum by 50% from the kidney by treatment with DMSA (550–1100 μmol/kg, i.p.) after CDDP (20 μmol/kg, i.v.) administration.[25] The doses of dimercapto-compounds used in their experiment were approximately 5–10 times higher than that in our experiment. Moreover, the dose of CDDP used in our experiment was therapeutically relevant and less than in other researches. Practically, the total dose (for 4 days) of CDDP was approximately 2.7 fold of intravenous dosage recommended in the guideline for cisplatin dosage (for 5days) in Japan, while it was about one third or a half in other experiments.[25,26] Furthermore, Graziano et al[25] presented that the treatment of DMSA more than 3 h after CDDP failed to prevent renal toxicity. Planas-Bohne et al[26] also referred to the necessity to select a reasonable time for

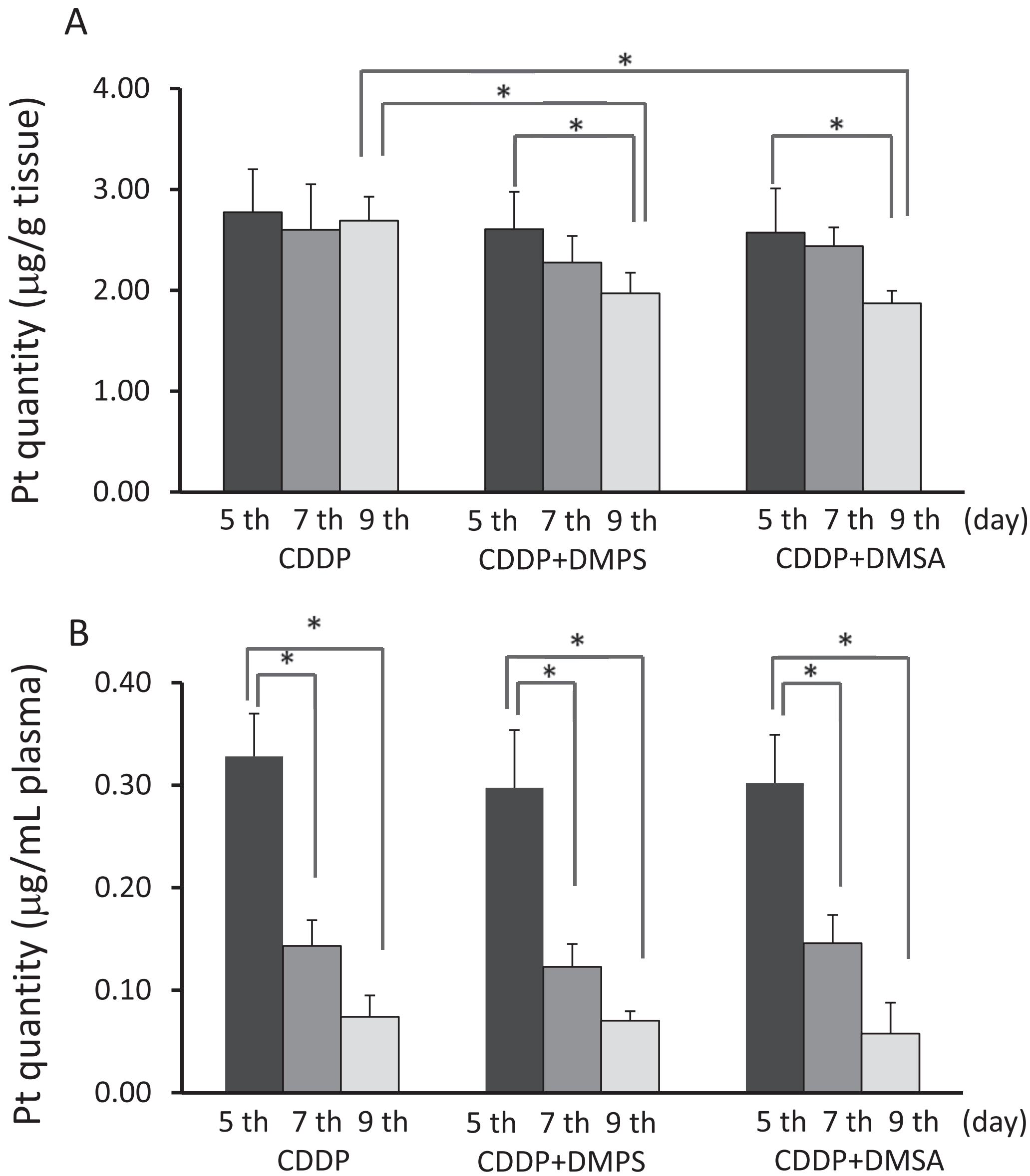

Fig. 5. Platinum accumulation in kidney and plasma. Platinum accumulation in kidney (A) and plasma (B) on the day 5, 7, or 9 after CDDP 5.7 μmol/kg with or without DMPS, DMSA 100 μmol/kg. Data are the mean ± S.D. of 6 mice per group. Statistically significant differences (*$p < 0.05$) are indicated.

between CDDP injection and the start of treatment for DMPS and DMSA. Those deduced that optimal order and time interval for treatment of chelating agents was necessary to elicit a more excellent effect to CDDP detoxification. In our experiment, the 1 h time interval between the start of pretreatment with chelating agents and CDDP was critical for effectively detoxifying CDDP-induced nephrotoxicity. Thus, our experiment was characterized by distinctive experimental design as follows: therapeutically relevant dose of CDDP; lower doses of DMPS and DMSA; pretreatment with DMPS and DMSA 1 h before CDDP administration.

Then, to certify that DMPS exerts not only enhancement of CDDP-induced antitumor activity but also reduction of CDDP-induced nephrotoxicity, we examined whether the lower doses (50, 100, 250 μmol/kg) of DMPS or DMSA effectively reverse the nephrotoxicity caused by CDDP (5.7 μmol/kg). Against the BUN levels raised by CDDP, DMPS reduced them in a time- and a dose-dependent manner with complete normalizing at 100 μmol/kg on 7th day, while DMSA reversed by 5th day at all doses. From these results, DMPS, as well as DMSA, seems to be an available agent for relief of nephrotoxicity. CDDP toxicity has been attributed to a number of molecular processes as DNA damage,[30,31] mitochondrial damage,[7,32] caspase activation,[8,33] formation of reactive oxygen species,[34,35] apoptosis and necrosis.[36–38] Some of researches reported that CDDP-induced renal injury was resulted from CDDP-generated apoptosis. That is, the cause of the increase in BUN was considered to be due to renal tubule cell death brought about by apoptosis. Therefore, it is very meaningful to investigate the effects of dimercapto-compounds on CDDP-induced apoptosis. Apoptosis in the kidney was assessed by using the TUNEL assay. Total dose of 22.8 μmol/kg of CDDP slightly increased the number of apoptotic nuclei compared with the control group. DMPS and DMSA at 100 μmol/kg completely suppressed CDDP-induced apoptotic degeneration on the nuclei of renal tubular cells. Thus, it was demonstrated that both dimercapto-compounds sufficiently and efficiently alleviate CDDP-induced renal failure even at lower doses. Furthermore, these findings strongly supported our tentative theory that DMPS at lower dose exert protective actions on the CDDP-induced nephrotoxicity. So, we obtained interesting findings on the kinetics of platinum (CDDP) in the kidney and plasma. In plasma, platinum concentration in each group of CDDP with/without DMPS or DMSA showed remarkable decrease in a time-dependent manner with one fifth-fold less on 9th day than on 5th day in all groups. Moreover, DMPS and DMSA gave no influence on enhancement of decreasing platinum in plasma. In the kidney, on the contrary, DMPS and DMSA promoted excretion of platinum, although with a little efficacy and with slow velocity, remaining 70–80% levels of platinum on 9th day comparing with on 5th day. Consequently, it is noteworthy that although quite a bit of platinum remained in the kidney of mouse pretreated with DMPS or DMSA, the renal function (BUN levels) was almost entirely reversed (as shown in the results of BUN levels), and was relieved from intoxication of CDDP. Transport system on the renal tubular cell membrane involved in the uptake and extrusion of drugs or other metabolites was provided by several researches: a permeable glycoprotein (P-gp) ABC family and organic ion transporter subtypes such as organic anion transporter (OAT1, 2, 3, OAT-K1), organic anion transporting polypeptide (OATP-1), OCT2, and MATE family. Of these transporters, OCT2 and MATE1 are predominantly involved in transport of CDDP, and contribute to uptake into and extrusion out of cells, respectively.[15] CDDP suppressed the expression of mRNA of MATE1 but a little on OCT2. On the other hand, DMPS and DMSA completely returned them to the normal level. These transporters have specificity to transporting organic cations exclusively. Then, CDDP as an inorganic cation was not easily transported via OCT2, an exclusive transporter for organic cations. Those things indicated that both transporters slightly contributed to transcellular movement of platinum in the kidney. These results indicated that although transporter regained normal function, CDDP was not effectively extruded from the kidney, but the velocity was very slow. Yokoo[39] proposed that CDDP as an inorganic cation is hard to be transported via OCT2, because of specificity of exclusively transporting organic cations. So, from our findings, it is speculated that CDDP retained in tissue is inactivated by forming complex with DMPS or DMSA to be nontoxic state. Moreover, CDDP-DMPS and -DMSA will be chemical forms easily to be transported through OCT2 and MATE1.

Verschraagen et al[40] presented that the high concentration of mercapto-compound (monothiol ligand) in the kidney inactivated CDDP by forming nontoxic platinum complexes. In general, compounds including dithiol ligand like DMPS or DMSA, possessing stronger coordination binding force, favor to react with heavy metals and result in forming CDDP-DMPS and CDDP-DMSA adducts. Accordingly, it has been thought to be theoretically proper that DMPS and DMSA greatly contributed to form the inactive and nontoxic platinum complexes, moreover, DMPS-CDDP or DMSA-CDDP adducts formed in the cells neutralized the nephrotoxicity of CDDP. Thus, it is indicated that lower dose of DMPS greatly helps not only increment of CDDP-induced antitumor activity but also decrement of CDDP-induced renal failure. Further investigations will need to be carried out to understand the extracellular excretion mechanism of complex compounds via transporter or membrane transport and the pharmacokinetics of intracellular CDDP and DMPS. We conclude that DMPS administered under the adequate condition on the dose and the time interval to CDDP exerts the effects not only enhancing antitumor activity but also reducing nephrotoxicity.

Conflict of interest

None.

Acknowledgments

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